

## In Vitro Monitoring of *Plasmodium falciparum* Drug Resistance in French Guiana: a Synopsis of Continuous Assessment from 1994 to 2005<sup>▽</sup>

Eric Legrand,<sup>1</sup> Béatrice Volney,<sup>1</sup> Jean-Baptiste Meynard,<sup>2</sup>  
Odile Mercereau-Puijalon,<sup>3</sup> and Philippe Esterre<sup>1\*</sup>

Reference Centre for *Plasmodium* Chemoresistance in French Guiana and West Indies (CNRCP), Institut Pasteur de la Guyane, Cayenne, French Guiana<sup>1</sup>; Epidemiology Unit, Institut Pasteur de la Guyane, Cayenne, French Guiana<sup>2</sup>; and Parasite Molecular Immunology, CNRS URA 2581, Institut Pasteur, Paris, France<sup>3</sup>

Received 21 February 2007/Returned for modification 2 May 2007/Accepted 13 October 2007

Implemented as one arm of the malaria control program in French Guiana in the early 1990s, our laboratory has since established in vitro profiles for parasite drug susceptibility to a panel of eight antimalarials for more than 1,000 *Plasmodium falciparum* isolates from infected patients. The quinine-doxycycline combination was introduced in 1995 as the first-line drug treatment against uncomplicated *P. falciparum* malaria, replacing chloroquine, and the first-line drug combination was changed to the artemether-lumefantrine combination in 2002. Resistance to chloroquine declined 5 years after it was dropped in 1995 as the first-line drug, but unlike similar situations in Africa, there was a rapid halt to this decline. Doxycycline susceptibility substantially decreased from 2002 to 2005, suggesting parasite selection under quinine-doxycycline drug pressure. Susceptibility to mefloquine decreased from 1997 onward. Throughout the period from 1994 to 2005, most isolates were sensitive in vitro to quinine, amodiaquine, and atovaquone. Susceptibility to amodiaquine was strongly correlated with that to chloroquine and to a lesser extent with that to mefloquine and halofantrine. Susceptibilities to mefloquine and to halofantrine were also strongly correlated. There were two alerts issued for in vitro artemether resistance in the period from 2002 to 2003 and again in 2005, both of which could be associated with the presence of an S769N polymorphism in the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)-type *P. falciparum* ATPase6 (PfATPase6) gene. Analysis of susceptibility to lumefantrine, conducted for the first time in 2005, indicates an alarming rate of elevated 50% inhibitory concentrations. In vitro monitoring of parasite drug susceptibility should be pursued to further document the consequences of specific drug policies on the local parasite population and, in particular, to establish profiles of susceptibility to individual components of drug combinations to provide early warning signs of emerging parasite resistance.

The Guyana Shield currently has the highest malaria rates in South America and is the only geographical area in the Americas where *Plasmodium falciparum* infection is diagnosed more frequently than *P. vivax* infection. In French Guiana, *P. falciparum* accounts for 60 to 70% of the 3,000 to 5,000 malaria cases reported every year (3). Malaria occurs in isolated geographical foci situated along the rivers in the Amazonian forest, and the population has little specific immunity. Malarial control involves spraying insecticides over the coastal region, where approximately 80% of the population resides, and using bed nets, an evidence-based drug policy, and deploying health services in remote endemic areas to provide prompt treatment by public primary health centers and/or private practitioners.

As in the neighboring countries of the Amazon basin (36), *P. falciparum* is multidrug resistant, with failures documented for chloroquine (18), the amodiaquine/sulfadoxine-pyrimethamine combination (22), halofantrine (9, 19), chloroquine-proguanil (19), and even quinine (6). In the last decade, the drug policy in French Guiana has been based on both an in vivo assessment of

therapeutic efficacy and longitudinal monitoring of in vitro drug susceptibility. In 1995, the quinine-doxycycline combination was recommended as the first-line treatment. It was replaced by the artemether-lumefantrine combination in 2002.

Monitoring drug resistance is particularly important in this region, where illegal gold-mining activities in the malaria endemicity areas are associated with erratic consumption of antimalarials and frequently with self-medication, unclear compliance to recommended regimens, and the formulation and use of illegally imported molecules of unknown quality, which contribute to the selection of drug-resistant parasites in the region (11, 29). However, surveillance of the therapeutic efficacy of the recommended regimens is complicated by the difficulty of monitoring treatment efficacy in the scattered endemicity foci. In vitro susceptibility testing by a dedicated reference laboratory was established in the early 1990s to carry out systematic in vitro drug susceptibility testing in French Guiana. In vitro tests assess parasite susceptibility to the individual components of drug combinations and, as such, provide early evidence of emerging resistance before it becomes clinically apparent. A recent illustration was the first description by our group of parasites from French Guiana with a markedly reduced in vitro susceptibility to artemether (11, 17).

Our reference laboratory has monitored, on a longitudinal basis, the in vitro drug susceptibility of isolates collected across

\* Corresponding author. Mailing address: CNRCP, Institut Pasteur de Guyane, PO box 6010, 97306 Cayenne Cedex, French Guiana. Phone: 594 594 292612. Fax: 594 594 318083. E-mail: cnrcp@pasteur-cayenne.fr.

<sup>▽</sup> Published ahead of print on 22 October 2007.



FIG. 1. Map of French Guiana. The main endemicity areas are highlighted in red. Solid zones indicate areas of high-incidence transmission, and hatched zones are low-incidence transmission areas.

the endemicity areas over the last 11 years. We have assessed parasite susceptibility to a panel of eight antimalarials, including the recommended drugs and molecules that are not yet in use in the area or that have been withdrawn. We report here a synopsis of the temporal variations in *in vitro* susceptibility profiles over that time period. We looked for temporal trends for each individual molecule, for cross-resistance between drugs from the same chemical class or between drugs with similar modes of action, and for correlations between susceptibility to different drug classes. We discuss these data in light of accumulated drug pressure and changes in drug policies in French Guiana and in the Guyana Shield region.

#### MATERIALS AND METHODS

Background information on the study area, clinical failure rates, and drug

policy. French Guiana (administratively a French Overseas Department) has nowadays an overall population of approximately 150,000 inhabitants. The main malaria endemicity areas are shown in Fig. 1. Malaria occurs in isolated, remote geographical foci located mainly along the Maroni River to the west and along the Oyapock River to the east, which serve as natural frontiers with Suriname and Brazil, respectively. Annual parasitic indices increase from the delta region (72 and 64‰ for the lower Maroni and Oyapock rivers, respectively) to the upper-river regions (371 and 223‰ for the upper Maroni and Oyapock rivers, respectively), where illegal mining activities occur (8).

There was a 22% rate of level RIII chloroquine resistance in 1989 (9, 18). The first sulfadoxine-pyrimethamine-resistant parasites were identified in 1988 (22). The combination was rapidly discontinued thereafter. There was an 11% clinical failure rate for the amodiaquine/sulfadoxine-pyrimethamine combination in 1986 (22). Halofantrine treatment failures, reported in the Maroni River area since 1988 (19; C. Venturin, I. Jeanne, J. B. Duchemin, G. Desmarchelier, V. Pavec, S. Laventure, E. Nuiaouet, J. Sankale-Suzanon, and J. L. Sarthou, presentation at the 3rd Infectious Diseases Medical Conference in French Guiana, Cayenne, 1988) reached 14% in 2000 (9). Two cases of level RIII *in vivo*

TABLE 1. Antimalarial regimens recommended for French Guiana by the 1990, 1995, and 2002 consensus meetings

Yr of consensus meeting	Clinical form	Therapy		Prophylaxis
		First-line	Second-line	
1990	Mild	Chloroquine <sup>b</sup>	Halofantrine <sup>c</sup> or mefloquine <sup>d</sup>	Mefloquine <sup>e</sup>
	Severe	Quinine <sup>a</sup>		Chloroquine-proguanil <sup>f</sup>
1995	Mild	Quinine-doxycycline <sup>h</sup>	Halofantrine <sup>c</sup> or mefloquine	Mefloquine <sup>e</sup> or doxycycline <sup>j</sup>
	Severe	Quinine-doxycycline <sup>g</sup>		
2002	Mild	Artemether-lumefantrine <sup>k</sup>	Atovaquone-proguanil <sup>l</sup>	Atovaquone-proguanil <sup>m</sup>
	Severe	Quinine-doxycycline <sup>g</sup>		Doxycycline <sup>j</sup>

<sup>a</sup> Total dose of 24 to 30 mg quinine base/kg of body weight.

<sup>b</sup> Forty milligrams per day for 4 days.

<sup>c</sup> Five hundred milligrams, 3 times per day.

<sup>d</sup> 1.5 g, 3 times per day.

<sup>e</sup> Two hundred fifty milligrams per week until 4 weeks after the patient's return, if <1-month stay.

<sup>f</sup> Chloroquine, 5 mg/kg per week, plus proguanil, 3 mg/kg per day, until 6 weeks after the patient's return, if 1- to 3-month stay.

<sup>g</sup> Quinine, 20 mg/kg intravenously for 4 h, and then 10 mg/kg/8 h, plus doxycycline, 3 mg/kg.

<sup>h</sup> Quinine, 25 mg/kg for 5 days, plus doxycycline, 3 mg/kg for 5 days.

<sup>i</sup> 24 mg/kg, 3 times per day.

<sup>j</sup> 100 mg per day for adults, until 4 weeks after the patient's return.

<sup>k</sup> Artemether, 20 mg, plus lumefantrine, 120 mg, 4 tablets of each 6 times per day.

<sup>l</sup> Atovaquone, 250 mg, plus proguanil chlorhydrate, 100 mg, 4 tablets of each per day for 3 days.

<sup>m</sup> 1 tablet per day until 7 days after the patient's return.

resistance to quinine have been identified (6). From the 1980s onward, quinine was always used in association with tetracycline (similar to protocols used in Brazil and Suriname) and not with sulfadoxine-pyrimethamine, as used in Guyana. Prophylaxis with chloroquine-proguanil remained clinically effective in French Guiana until 1998, when seven falciparum malaria attacks, including two severe forms, occurred on a military base where chloroquine-proguanil prophylaxis was given (19).

A "consensus conference" was organized at 5- to 7-year intervals in order to review in vivo and in vitro drug susceptibility in the area and to adjust the treatment policy accordingly. Table 1 summarizes the treatments recommended for mild malaria, severe malaria, and prophylaxis from 1990 to 2006. From 1990 to 2005, the first-line and second-line treatment drugs were chloroquine and halofantrine or mefloquine, respectively. The 1995 consensus conference introduced the quinine-doxycycline association as the first-line treatment. The update at the end of 2002 replaced this combination with atovaquone-proguanil (mainly for prophylaxis) and artemether-lumefantrine (for therapy) (Table 1). The artemether-lumefantrine combination (Riamet; Novartis) is currently the recommended first-line drug for the treatment of uncomplicated falciparum malaria in adults in French Guiana, but it has not yet been approved for use in children.

The antimalarials used are prescribed by doctors when patients seek treatment (from private doctors, public health centers, or hospitals). The medical staff is urged to follow the regional guidelines of the Ministry of Health. However, an unknown proportion of malaria cases occur in the illegal immigrant population in the area, mainly gold miners operating in the jungle. These "illegal" cases are treated mostly by self-medication and are prevented by self-prescribed prophylaxis with cheap drugs from neighboring Brazil and Suriname.

**Isolates: geographic origin and patients.** All isolates studied were collected from malaria patients referred to our reference laboratory by private practitioners, health centers, and/or hospitals, as recommended by the health authorities. A 2- to 5-ml blood sample was collected from each patient by venipuncture in EDTA tubes, before they received treatment administration/prescription. The tube was maintained at 4°C during shipment to the regional reference center in Cayenne (CNRCP), where it was immediately processed by using in vitro susceptibility assays.

Out of the 2,855 isolates originating from the peripheral health centers located along the Maroni (60% of the annual sampling) and the Oyapock rivers and from the main hospitals on the coast, mainly in Cayenne (30%) and Saint-Laurent (9%), 2,535 isolates were identified as a monospecific *P. falciparum* infection.

**In vitro susceptibility assays.** The inclusion criteria for in vitro drug assays were (i) a microscopically diagnosed (thin smear) *P. falciparum* isolate-monospecific infection (i.e., the absence of concomitant *P. vivax* and *P. malariae* isolates), confirmed in some cases by an immunochromatographic dipstick test or PCR, and (ii) a specimen collected less than 3 days before laboratory processing and before treatment administration. The samples were processed on receipt at the CNRCP and tested for susceptibility to eight antimalarial drugs, using a modified [<sup>3</sup>H]hypoxanthine-uptake in vitro assay (14) and a computer-piloted

(GX110 model Del) multidetector scintillation counter (Microbeta Trilux; Wallac Oy, Turku, Finland).

Tests were carried out over a 48-h culture period, using the [<sup>3</sup>H]hypoxanthine incorporation microtest. Each isolate was tested once in triplicate against serial twofold drug dilutions over the concentration ranges of 3,750 to 7 nM chloroquine (diphosphate; Sigma catalog no. C6628), 3,680 to 7 nM quinine (sulfate; Sigma catalog no. Q1878), 793 to 1.54 nM mefloquine (Hoffman-La Roche Inc), 63.1 to 0.123 nM halofantrine (GlaxoSmithKline Inc.), 840 to 1.64 μM amodiaquine (dihydrochloride dihydrate; Sigma catalog no. A2799), 50 to 0.097 μM cycloguanil (Astra Zeneca), 1039 to 2.03 μM doxycycline (hydrochloride; Sigma catalog no. D9891), 98.5 to 0.19 nM artemether (Novartis Pharma Inc), and 32 to 0.00625 nM atovaquone (GlaxoSmithKline Inc). The various antimalarial molecules were tested from the beginning, except for amodiaquine (for which testing began in 1995), artemether (in 1997), atovaquone (in 2001), and lumefantrine (in 2005).

Control susceptible lines were included for quality control. The 3D7 clone was used from 1999 onward. In 2002, we also included HB3 and W2, and 7G8 was added to the panel of control lines in 2003. The four control lines were kindly provided by J. Le Bras (Centre National de Référence pour la chimiosensibilité du paludisme, Paris, France). *P. falciparum* reference lines were run in parallel assays for each batch of antimalarial used. Since 1999, 3D7 has been tested in 13 assays; and HB3, W2, and 7G8 were tested in 12, 7, and 5 independent assays, respectively. The 50% inhibitory concentration (IC<sub>50</sub>) was calculated using a probit/logit regression of the percentage of growth inhibition for each drug, and the geometric mean IC<sub>50</sub>s were compared by Student's *t* test. To comply with previous reports from the area and with the commonly used interpretation of susceptibility tests (5, 14, 15, 20, 26, 28, 29), we have used a cutoff value to classify isolates as susceptible, intermediate, or resistant (Table 2). The in vitro levels of chloroquine resistance versus sensitivity were determined at the laboratory level by analyzing isolates from clinical cases with documented therapeutic efficacy from the area. For the antimalarials other than chloroquine, there is no threshold established for these grounds. It is not possible to assign such a value for drugs not yet in use in the area (or no longer in use). The temporal variation of in vitro-resistant prevalence was followed in the various areas. Generally speaking, the Maroni River samples (from two sentinel sites, Maripasoula and Saint-Laurent du Maroni), representing isolates from a region with a high prevalence of multidrug resistance, were compared with those from the rest of the department.

**DNA extraction and microsatellite genotyping.** The artemether-resistant sample isolated in 2005 was cultured for 21 days with the absence of artemether. DNA was prepared from the ex vivo sample and, after 21 days of culture, as described previously (25), was genotyped for four microsatellites, DHFR, PFE14F, C3M35, and C4M79, as described previously (16). The full-length *P. falciparum* *ATPase6* (PfATPase6) gene was amplified for both samples and was sequenced as described previously (11).

**Statistical analysis.** The global trends were calculated using a Kruskal-Wallis test for the last 6 years. In addition, the year-to-year evolution was analyzed using



TABLE 2. Cutoff used by the reference laboratory for interpretation of in vitro susceptibility assays

Molecule	IC <sub>50</sub> cutoff value (nM) used to define an isolate as:			Reference(s)
	Susceptible	Intermediate	Resistant	
Chloroquine	<80	80–100	>100	2, 5, 28, 29
Monodesethyl amodiaquine	<40	40–60	>60	5, 28, 29
Quinine	<300	300–500	>500	5, 28
Mefloquine	<15	15–30	>30	2, 28, 29
Halofantrine	<4	4–8	>8	1, 28, 29
Doxycycline			>9.6 <sup>a</sup>	29
Atovaquone	<5	5–7	>7	29
Artemether			>12; >30 <sup>b</sup>	10, 26, 28

<sup>a</sup> For doxycycline, the IC<sub>50</sub> is expressed in  $\mu$ M.

<sup>b</sup> Defined as the mutant isolate with the *PlATPase6* gene S769N polymorphism.

Student's *t* test with a level of significance at a *P* value of <0.05. In vitro correlations between the mean IC<sub>50</sub>s of various drugs were tentatively searched by means of standard correlation analysis (Pearson's test, *P* < 0.05) on a significant sample of isolates (*n* = 171 and *n* = 511, collected during the periods from 1996 to 1999 and 2000 to 2005, respectively). All analyses were carried out by using specialized software (SAS version 9.1; Sas Institute Inc., Cary, NC).

## RESULTS

**Chloroquine.** Chloroquine was the first-line drug for treating *P. falciparum* malaria until 1995, when its use was discontinued. From 1994 to 1999, close to 100% of the isolates were classified as in vitro chloroquine resistant (Fig. 2A). The geometric mean IC<sub>50</sub> was high ( $\geq 400$  nM). From 2000 on, there were abrupt reductions in both the proportion of chloroquine-resistant isolates and the geometric mean IC<sub>50</sub>s, which dropped from 371 nM in 1999 to 96 nM in 2000 (*t* test, *P* < 0.0001). However, there was no further decline in subsequent years. Geometric mean IC<sub>50</sub>s increased moderately from 98.1 nM in 2001 to 107 nM in 2002, 120.2 nM in 2003, 124.5 nM in 2004, and 100.1 nM in 2005 (Kruskal-Wallis test, *P* = 0.01).

**Amodiaquine.** Amodiaquinized salt (0.43%) was distributed to the whole population from 1967 to 1978 (and in neighboring Suriname from 1965 to 1973) (27) and sporadically on an individual level at later dates (8). Amodiaquine susceptibility was tested routinely from 1996 onward. Approximately 94% of 482 isolates tested from the period from 1996 to 2005 were sensitive to amodiaquine (Fig. 2B). There has been, however, an increase in mean IC<sub>50</sub>s in recent years, rising from 11.3 nM in 2000 to 28.2 nM in 2004 and to 22.3 nM in 2005 (Kruskal-Wallis test, *P* < 0.0001).

**Quinine.** Quinine has been used for the treatment of severe or complicated cases in combination with tetracycline since the 1980s. The quinine-doxycycline association was introduced as the first-line drug for treating *P. falciparum* malaria in 1995. An elevated IC<sub>50</sub>, classified as quinine resistance, was reported for 17% of isolates from 1983 to 1987 (5). This classification accounted for 9 to 12% of the isolates analyzed from 1995 to 1997 and was no longer observed for 487 isolates tested from 2000 to 2005, with the exception of two geographically localized alerts issued over the last 3 years (Fig. 2C). All during the period surveyed, the yearly mean IC<sub>50</sub> remained in the sensitive zone (196.8 nM for 1998 to 2005), albeit with a significant

increase from 1995 to 2001, followed by a significant decrease from 2001 to 2005 (Kruskal-Wallis test, *P* < 0.0001).

**Mefloquine.** As shown in Table 1, mefloquine was used for prophylaxis and as a second-line treatment drug throughout the years 1990 to 2002. In the 1980s and until 1995, all isolates tested were susceptible to mefloquine. In 1996, 2% of the isolates were classified as in vitro mefloquine resistant. The prevalence of mefloquine resistance subsequently fluctuated between 0 and 29% (Fig. 3A). From 1997 onward, isolates with "intermediate" levels accounted for 11 to 23% of the isolates tested. The mean IC<sub>50</sub> increased during this time period, from 9.4 nM in 1998 to 20.9 nM in 2002 and to 13.5 nM in 2005 (Kruskal-Wallis test, *P* = 0.039). It is interesting to note that the temporal variations were reflected at both the global and the local (Maroni River area) levels.

**Halofantrine.** From 1994 to 2005, most isolates were classified as sensitive, and a minor fraction (6 to 9%) presented intermediate levels of resistance. This proportion increased to 21% in the next 2 years and fluctuated thereafter between 14 and 34% (Fig. 3B). Isolates classified as in vitro halofantrine resistant were observed in 1996 and in subsequent years, but their prevalence fluctuated markedly. There was a peak of 35% prevalence of resistance for isolates from 1997 and a second peak of 66% prevalence of resistance in isolates from 2000. It decreased to less than 3% in 2004. The mean IC<sub>50</sub> increased from 3 nM in 1997 to 14.1 nM in 2000 and decreased significantly thereafter (Kruskal-Wallis test, *P* < 0.0001) to 3.8 and 4.1 nM in 2004 and 2005, respectively.

**Doxycycline.** Doxycycline has been investigated since mid-1996, using standard 48-h tests, despite the fact that it is a delayed parasite death inducer. With this limitation in mind, we nevertheless reviewed the data available across the decade. As there was no global consensus for the threshold of susceptibility, a tentative cutoff level was set at 9.6  $\mu$ M by Sarthou and Reynes (29), corresponding to the 95th percentile of a representative local sample. The prevalence of isolates with an IC<sub>50</sub> above that threshold rose from 15 to 25% in the period from 1996 to 2001, to 51% in 2002, to 61.5% in 2003, and to more than 67% in 2005 (Fig. 4). Mean IC<sub>50</sub>s increased from 9.6  $\mu$ M in 1996 to 1999 to 7.4  $\mu$ M in 2000, 12.2  $\mu$ M in 2002 to 2003, and 13.1  $\mu$ M in 2005 (Kruskal-Wallis test, *P* < 0.0001).

**Proguanil/cycloguanil.** Resistance to proguanil, based on in vitro testing of its major metabolite cycloguanil, had a very high prevalence (98% in 1997 and 100% in all subsequent years) and was at an elevated level (mean IC<sub>50</sub>s, >4, 100 nM) (data not shown). In vitro testing was stopped in 2000.

**Artemisinin derivatives.** Artemether has been incorporated in our panel of antimalarials for in vitro testing since 1997. The great majority of 634 isolates assayed (95.5% to 98%) had a low IC<sub>50</sub> value. The mean IC<sub>50</sub> varied between 2.2 and 3.9 nM, except in 2002 when it was 8.4 nM (Fig. 5A), due to the presence of isolates showing markedly decreased in vitro sensitivity.

Figure 5A shows the yearly distribution of isolates grouped into two classes using the 12 nM threshold, which represents the lower border of the highest 5th percentile of IC<sub>50</sub> distribution among African isolates, as proposed by Pradines et al. (26). Figure 5B shows the distribution into two classes using the 30 nM threshold, which has been associated with the presence of a S769N polymorphism in the putative target enzyme sarco-

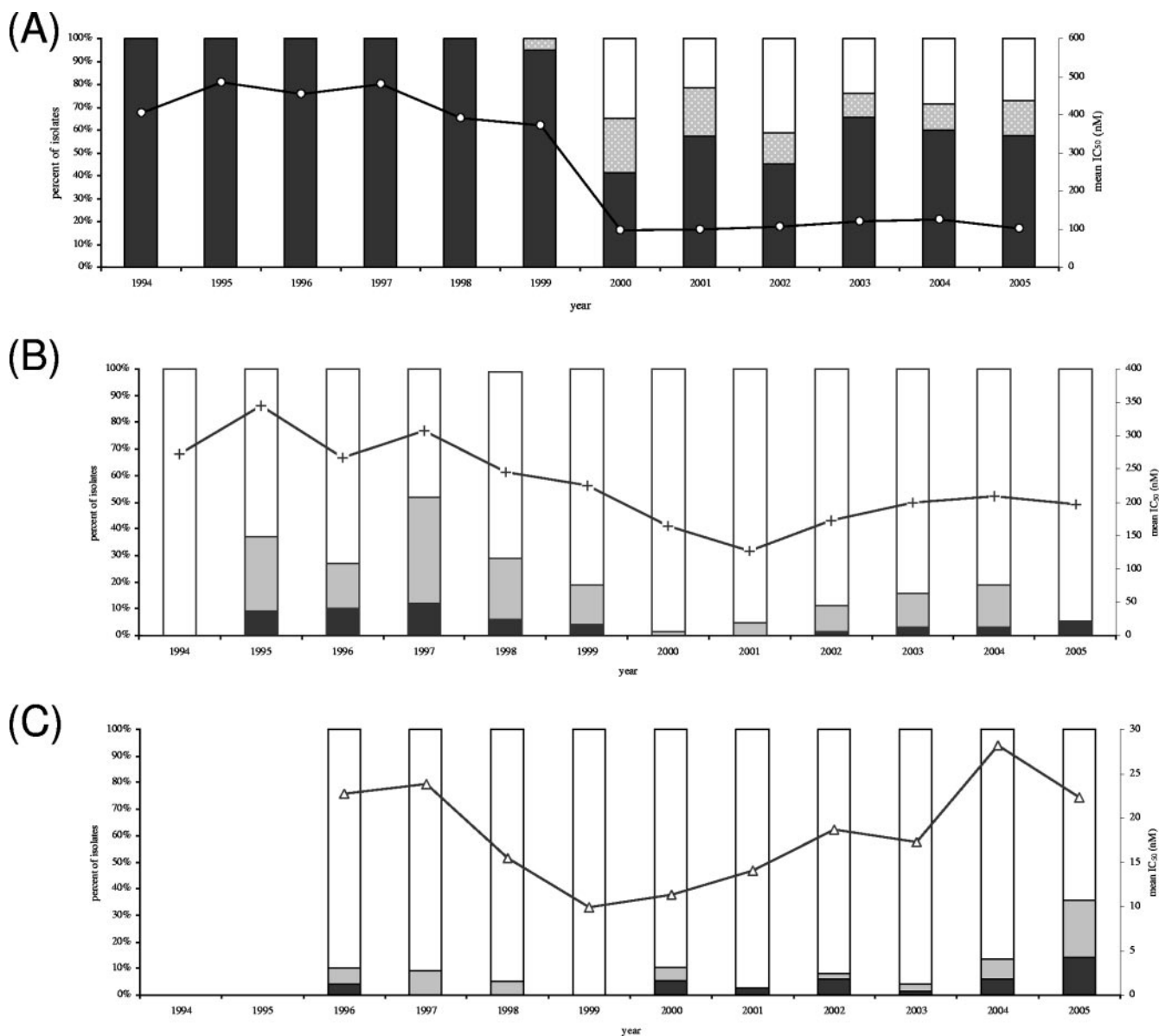


FIG. 2. Temporal evolution of the in vitro susceptibility to chloroquine (A), quinine (B), and amodiaquine (C) in French Guiana from 1994 to 2005. (A) In vitro susceptibility to chloroquine was obtained for 16, 22, 48, 68, 46, 44, 68, 42, 144, 159, 70, and 62 isolates collected in 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, and 2005, respectively. Isolates severity: open bars, susceptible ( $IC_{50}$  values of  $<80$  nM); gray bars, intermediate ( $IC_{50}$  values  $\geq 80$  but  $<100$  nM); black bars, resistant ( $\geq 100$  nM). The mean  $IC_{50}$  values (nM) are indicated by open circles (right axis). (B) In vitro susceptibility to quinine was obtained for 16, 22, 48, 68, 47, 52, 68, 42, 146, 159, 69 and 59 isolates collected in 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004 and 2005, respectively. Open bars, susceptible ( $IC_{50}$  values of  $<300$  nM); gray bars, intermediate ( $IC_{50}$  values of  $\geq 300$  but  $<500$  nM); black bars, resistant ( $IC_{50}$  values of  $\geq 500$  nM). The mean  $IC_{50}$  (nM) values are indicated by crosses (right axis). (C) In vitro susceptibility to amodiaquine was obtained for 48, 67, 43, 38, 19, 39, 134, 145, 66 and 14 isolates collected in 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004 and 2005, respectively. Open bars, susceptible ( $IC_{50}$  values of  $<40$  nM); gray bars, intermediate ( $IC_{50}$  values  $\geq 40$  but  $<60$  nM); black bars, resistant ( $IC_{50}$  values  $\geq 60$  nM). The mean  $IC_{50}$  (nM) values are indicated by open triangles (right axis).

plasmic/endoplasmic reticulum calcium ATPase (SERCA)-type PfATPase6 gene (11). Nine isolates with an  $IC_{50}$  for artemether of  $\geq 30$  nM were observed in 2002, and one isolate was observed again in 2005 (17). In all these cases, the observation of isolates with elevated  $IC_{50}$ s for artemether is unlikely attributable to experimental culture artifacts, since unrelated patient isolates assayed on the same day exhibited low  $IC_{50}$  values for artemether. Molecular typing of the isolate collected in 2005,

which presented an  $IC_{50}$  of 127 nM for artemether, using microsatellite markers indicated that the ex vivo isolate contained two clonal types (Table 3) and two PfATPase6 alleles, an S769N mutant allele and a wild-type allele. After 3 weeks of in vitro cultivation under standard culture conditions, the mutant allele was no longer detected, and the  $IC_{50}$  for artemether had dropped to 5 nM (16), suggesting a poor fitness of the mutant in vitro.

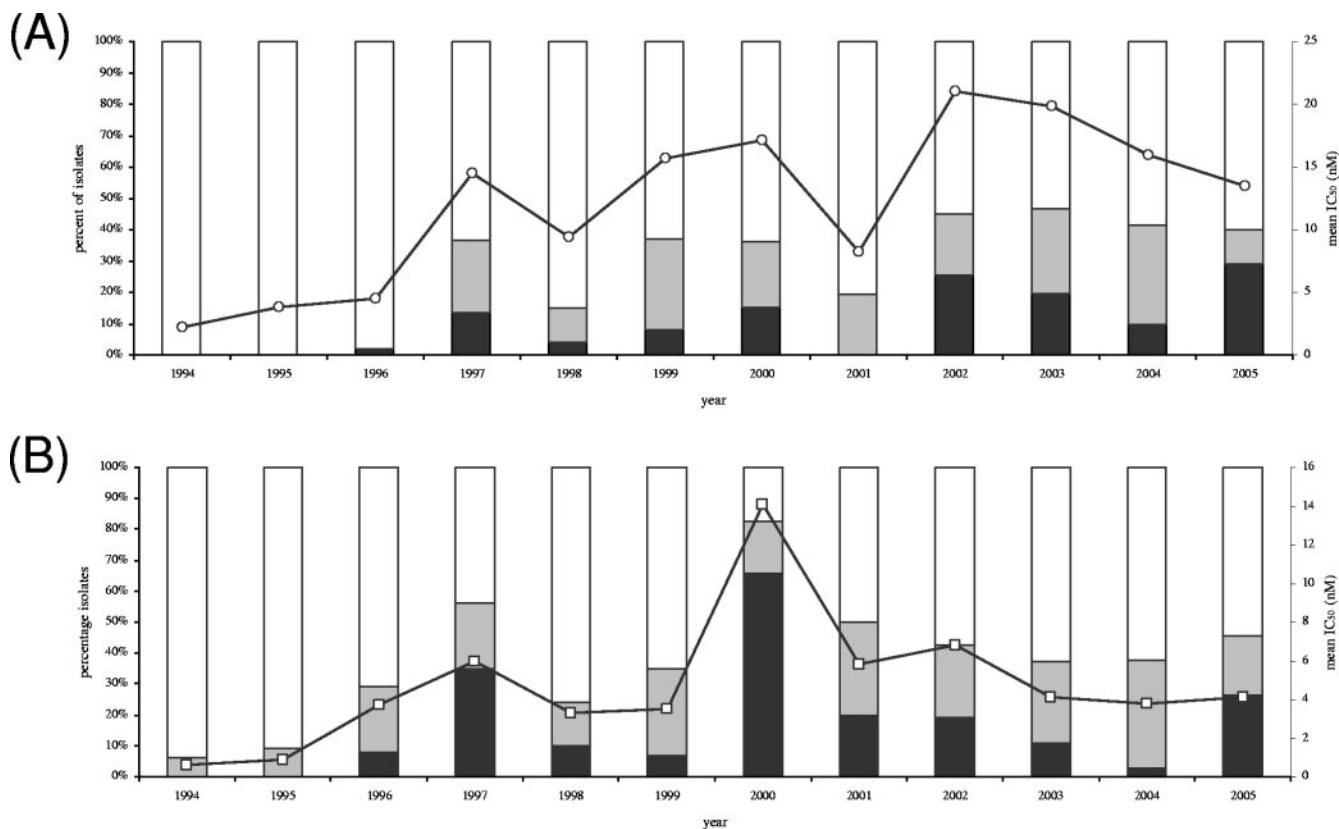


FIG. 3. Temporal evolution of in vitro susceptibility of French Guiana isolates to mefloquine (A) and halofantrine (B) from 1994 to 2005. (A) In vitro susceptibility to mefloquine was obtained for 16, 22, 43, 66, 45, 51, 66, 36, 142, 158, 70 and 58 isolates collected in 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004 and 2005, respectively. Open bars, susceptible ( $IC_{50}$  value of  $<15$  nM); gray bars, intermediate ( $IC_{50}$  values of  $\geq 15$  but  $<30$  nM); black bars, resistant ( $IC_{50}$  values  $\geq 30$  nM). The mean  $IC_{50}$  (nM) values are indicated by open circles (right axis). (B) In vitro susceptibility to halofantrine was obtained for 16, 22, 48, 68, 42, 46, 64, 40, 136, 156, 69, and 60 isolates collected in 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, and 2005, respectively. Open bars, susceptible ( $IC_{50}$  value of  $<4$  nM); Gray: intermediate ( $IC_{50}$  values of  $\geq 4$  but  $<8$  nM); black bars, resistant ( $IC_{50}$  values  $\geq 8$  nM). The mean  $IC_{50}$  (nM) values are indicated by open squares (right axis).

The distribution of  $IC_{50}$ s for artemether over the period from 2002 to 2005 is shown in Fig. 5C. The geometric mean  $IC_{50} \pm$  standard deviation during 2002 to 2005 was  $2.03 \pm 13$  ( $n = 413$  isolates). The interquartile range was 0.98 to 3.74.

Elevated  $IC_{50}$ s for artemether were observed for a very small fraction of all isolates.

**Lumefantrine.** The investigation of lumefantrine susceptibility in vitro was initiated in 2005. The distribution of  $IC_{50}$ s for

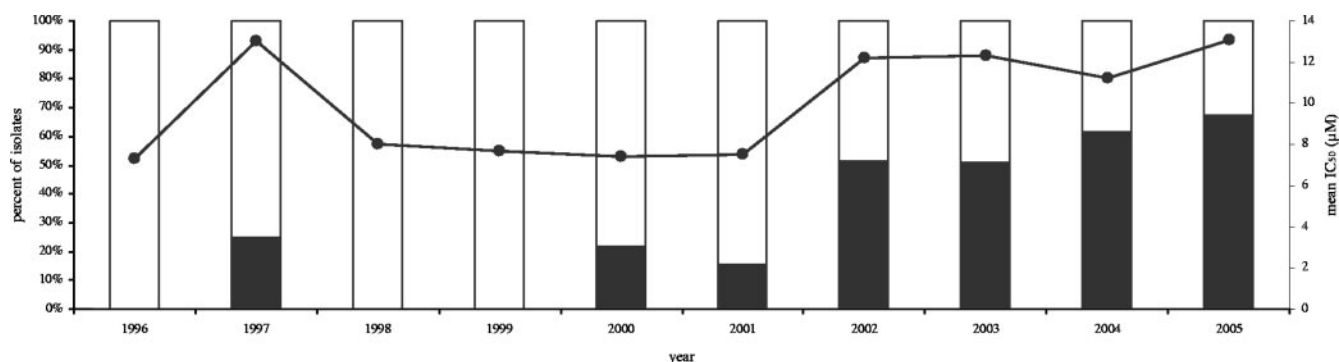


FIG. 4. Temporal evolution of in vitro susceptibility and mean  $IC_{50}$  values for doxycycline of French Guiana isolates assessed from 1996 to 2005. In vitro susceptibility to doxycycline was obtained for 34, 32, 45, 50, 69, 39, 134, 147, 65, and 55 isolates collected in 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, and 2005, respectively. Open bars, susceptible ( $IC_{50}$  values  $<9.6$   $\mu$ M); black bars, resistant ( $IC_{50}$  values  $\geq 9.6$   $\mu$ M). The mean  $IC_{50}$  ( $\mu$ M) values are indicated by closed circles (right axis).

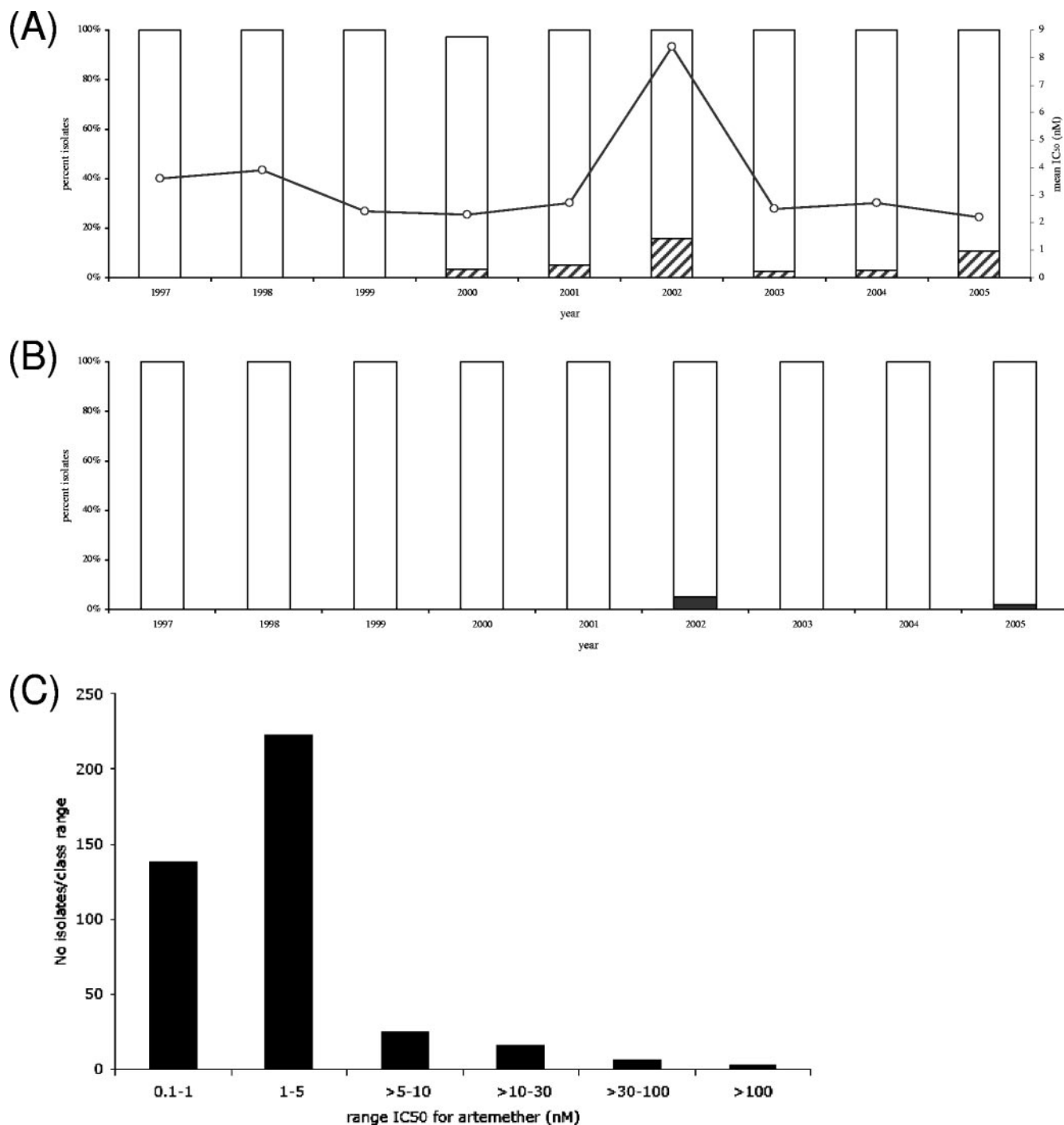


FIG. 5. Temporal evolution of in vitro susceptibility of French Guiana isolates to artemether from 1997 to 2005. In vitro susceptibility to artemether was obtained for 31, 42, 44, 65, 39, 136, 153, 66, and 58 isolates collected in 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, and 2005, respectively. Prevalence of isolates with an  $IC_{50}$  value above 12 nM (A) or the 30 nM (B) cutoff. The mean  $IC_{50}$  (nM) values are indicated by open circles in panel A (right axis). Frequency distribution by  $IC_{50}$  class range is shown in panel C.

lumefantrine, shown in Fig. 6, spanned a 3 log concentration range. This limited sample showed a geometric mean  $IC_{50} \pm$  standard deviation of  $156.8 \pm 534.6$  nM. Of 36 isolates tested, 14 (38.8%), 4 (11.1%), and 18 (50%) isolates presented  $IC_{50}$  values of <100, 100 to 150, and >150 nM, respectively, for this molecule.

**Atovaquone.** Atovaquone has been used in the area in association with proguanil (Malarone). The 368 in vitro tests performed before its implementation in the area (which started in 2002) showed an excellent level of sensitivity (with a mean  $IC_{50}$  for atovaquone of 0.55 nM) during the period from 1998 to 2004 (data not shown). The first case of in vitro resistance to

TABLE 3. Susceptibility test and genotype of artemether resistance isolate at day 0 and day 21<sup>a</sup>

Day	IC <sub>50</sub> (nM) for							Genotyping result					
	Artemether	Chloroquine	Quinine	Halofantrine	Lumefantrine	Mefloquine	Doxycycline	Microsatellite (bp)				% of isolates carrying PfATPase6	
								DHFR	PFE14F	C3M35	C4M79	Wild type	S769N mutant
0	127	67	32	1.3	20.9	2.4	5,800	96	132	207	210	54	46
											194		
21	8.2	75	28	2.1	35	3.2	7,500	96	132	207	194	100	

<sup>a</sup> Values for in vitro susceptibility tests and genotypes are shown for the artemether resistance strain ex vivo isolates collected in 2,005 and results with the same isolates after 21 days of continuous cultivation.

atovaquone was clinically and parasitologically documented in 2005 with parasites collected from a patient for whom treatment with second-line atovaquone-proguanil failed (15).

**In vitro cross-reactivity.** In vitro correlations between the mean IC<sub>50</sub>s for drugs with similar modes of action or for those of the same chemical classes were tentatively sought from two groups of samples collected from 1996 to 1999 and from 2000 to 2005. A lack of correlation between chloroquine and quinine, mefloquine, or doxycycline was noted. In contrast, positive correlations were observed between amodiaquine and chloroquine ( $r = 0.75$ ;  $P = 0.02$ ), amodiaquine and halofantrine ( $r = 0.51$ ;  $P < 0.001$ ), amodiaquine and mefloquine ( $r = 0.48$ ;  $P = 0.004$ ), and to a lesser extent amodiaquine and quinine ( $r = 0.25$ ;  $P = 0.03$ ).

There were highly significant correlations between mefloquine and halofantrine in isolates from 1996 to 1999 ( $r = 0.72$ ;  $P = 0.01$ ) and those from 2000 to 2005 ( $r = 0.63$ ;  $P < 0.0001$ ). Interestingly, similar correlations had been noticed during local surveys organized in the mid-Maroni River area (Papaich-ton,  $r = 0.74$ ,  $P < 0.01$ , on 27 in vitro tests analyzed in 2000 [9]) and in the high-Maroni River area (Maripasoula,  $r = 0.73$ ,  $P < 0.0001$ , on 29 tests in 1995 [C. Venturin et al., 3rd Infectious Diseases Medical Conference, 1988]). For lumefantrine, a significant correlation was noticed with halofantrine ( $r = 0.78$ ;  $P < 0.0001$ ), but there was no association with mefloquine.

## DISCUSSION

Our data highlight significant temporal changes of in vitro sensitivity to most molecules included in the established systematic screening scheme over the 12 years surveyed. Chloroquine and quinine susceptibility increased significantly during this period. Susceptibility to doxycycline, mefloquine, and amo-

diaquine decreased, and there were alerts issued for halofantrine and artemether resistance. These changes can be interpreted in light of the drug pressure exerted on the parasites in French Guiana and in the surrounding countries. Drug pressure in the area has been quite high from very early on. Amodiaquinized salt (0.43%) was distributed to the whole population from 1967 to 1978 (and in neighboring Suriname from 1965 to 1973) (30) and distributed sporadically on an individual basis after that (8). Chloroquine, quinine, tetracycline, doxycycline, mefloquine, and halofantrine have been used during the last decade, and more recently, atovaquone, proguanil, artemether, and lumefantrine have been introduced.

The use of chloroquine for treating *P. falciparum* malaria was abandoned in French Guiana in 1995 because of a documented loss of therapeutic efficacy at a local level and across the Amazonian region. During the following years, all French Guiana isolates tested were chloroquine resistant, but there was a marked increase in chloroquine sensitivity in 2000 and thereafter. The decrease in chloroquine resistance was altogether abrupt and partial. The abruptness is difficult to interpret and may reflect a geographical sample bias, as some endemic foci may have been overrepresented during certain years. As shown in Fig. 1, the endemic foci in French Guiana have a patchy distribution, are isolated, and are separated by large inhabited primary forests and/or river banks. This situation may be responsible for microgeographic parasite heterogeneity, including drug resistance, as well as heterogeneity of transmission intensity and hence for the spread of resistance. Whereas this may create distribution distortions during some periods, this proviso no longer holds for longer term trends, such as the shift from 100% chloroquine resistance in 1995 to 1999 to 50 to 60% resistance in 2000 to 2005. Similarly partial decreases in chloroquine-resistant *P. falciparum* isolates after chloroquine cessation were observed in China (21, 34) and in Cambodia (7, 20). These decreases differ from the consequence of chloroquine cessation in Malawi that was followed by a decrease in the Pfcr1 76T marker from 85% in 1993 to 13% in 2000 and 0% in 2001 (13, 23). The maintenance of substantial levels of chloroquine resistance in non-African settings after the discontinuation of chloroquine treatment against *P. falciparum* malaria reflects the fact that unlike the situation in Africa, not all chloroquine pressure has been removed from the region. In particular, chloroquine has been used continuously and is still being used for the treatment of *P. vivax* malaria, which accounts for a substantial fraction of malaria

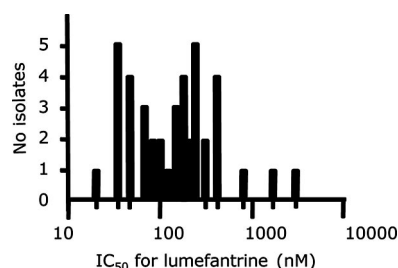


FIG. 6. Frequency distribution of IC<sub>50</sub> values for lumefantrine in French Guiana for 41 isolates collected during 2005.



cases and is most probably used also against some *P. falciparum*-*P. vivax* mixed isolate infections.

However, French Guiana treatment differs from that in Southeast Asian countries and China in the replacement of chloroquine. As in many countries in this region, in French Guiana chloroquine was replaced as the first-line treatment drug for *P. falciparum* malaria by the quinine-doxycycline combination. Whether this has contributed to preventing further decreases in chloroquine resistance is unclear. The decrease in chloroquine resistance is usually interpreted as due to its fitness cost in the absence of chloroquine pressure (33). Molecular analysis using transgenic parasites mutated at the *Pfcr*t or the *Pfmdr*1 locus showed an inverse relationship of mutations governing in vitro susceptibility to chloroquine on the one hand and to quinine, mefloquine, and halofantrine on the other hand in some genetic backgrounds (12, 13, 27, 31, 32). No such association was observed for the panel of isolates studied from French Guiana. In vitro susceptibility to chloroquine was associated with no other response than amodiaquine, consistent with the amodiaquine and chloroquine cross-resistance reported in many settings. Interestingly, amodiaquine susceptibility was associated with halofantrine, mefloquine, and to a lesser extent quinine susceptibility. Molecular studies of *Pfcr*t, *Pfmdr*1, and other putative chloroquine and quinine transporters are under way to understand the molecular basis of such associations and to document the consequences of changes in first-line treatment drugs for French Guiana parasites.

In French Guiana, as in Brazil and Suriname, quinine had been used for the treatment of severe or complicated cases of malaria in combination with tetracycline since the 1980s. The quinine-doxycycline combination was introduced for uncomplicated *P. falciparum* malaria cases in 1995. This combination has been used extensively in South America during the last 10 years and is still used because of irregular supplies of artemether-lumefantrine and nonprescription of artemether-lumefantrine in pediatric malaria cases. Our data indicate that the use of the quinine-doxycycline combination has selected parasites with reduced in vitro susceptibility to doxycycline. This may be underestimated by the use of 48-h in vitro assays since doxycycline is a slow-acting antimalarial. A more accurate assessment of doxycycline inhibition would have required cultivation for 72 to 96 h. Plans are under way to establish these assays in the laboratory. The reduction observed for in vitro susceptibility to doxycycline may have had limited clinical impact so far, in particular because of the continuing efficacy of quinine. However, further extensive use of doxycycline or of any combination drug containing doxycycline is not desirable, since it will further increase resistance to doxycycline and, in turn, to the combination in quinine monotherapy, thereby increasing the selection pressure for quinine resistance.

Artemisinin derivatives have been introduced in French Guiana in combination with lumefantrine (Riamet), but as discussed above, the treatment is not yet approved for treating pediatric malaria in French Guiana or for treating pregnant women and is not widely available. Furthermore, its elevated price is an incentive for users to cross the border and purchase artemether-lumefantrine (Coartem) and/or other artemisinin-based combination therapy drugs in neighboring countries. In the years 2002 and 2005, we observed isolates with markedly

increased  $IC_{50}$ s for artemether and a *PfATPase*6 S769N mutation. The mean  $IC_{50}$  for artemether ( $\pm$  95% confidence interval [CI]) with the *PfATPase*6 S769N mutant isolates was 82.6 nM ( $\pm$  95% CI, 23.3), i.e., 25-fold above the upper border of the 95% CI value for artemether for all parasites studied in French Guiana (geometric mean = 2.03 nM; 95% CI = 1.26). There are two points of concern here. First, these isolates originated from areas quite distant from each other across French Guiana, namely from the town of Cacao, and from settings along the Maroni River, which are separated by inhabited primary forest (Fig. 1). Epidemiological investigations of these patients revealed that drugs labeled as containing an artemisinin derivative had been illegally imported from Southeast Asia into Cacao. The exact drug composition and concentration could not be determined by the expert chemistry laboratory at the Institut de Veille Sanitaire. Whether this finding indicates counterfeit, underdosed drugs, which dramatically plague the market nowadays (24), is unclear. Investigations focus on areas along the Maroni River with illegal gold mining activities and the importation of antimalarial drugs from Suriname and Brazil, in particular dihydroartemisinin-piperaquine (Artekin [Tonghe Pharmaceuticals]) and piperaquine-dihydroartemisinin-trimethoprim (Artecom [Tonghe Pharmaceuticals]). The quality of these drugs has not been assessed, and the possibility of counterfeits remains open.

A second point of concern is the observation of an additional case of in vitro resistance to artemether from the Maroni River area, which was associated with exactly the same mutation in the target gene 3 years after the first cases. Unfortunately, this isolate could not be maintained in culture and could not be cloned out under artemether pressure in vitro after freeze-thawing, an experience also reported by another group (10). This suggests that prolonged cultivation can select for a drug-sensitive parasite, possibly because current culture conditions may be suboptimal for certain parasite strains. Since many field isolates are from mixed infections, this raises a warning that caution should be used when cultivating field isolates for extended periods of time. The data indicate that the emergence of resistance to artemether in the region is indeed a threat. Artemisinin derivatives are also active on gametocytes, thereby reducing the probability of resistance spreading. Nevertheless, continued and increased surveillance is needed to prevent the establishment of isolates with additional mutations. The S769N polymorphism in the *PfATPase*6 gene may represent a first step in a process of accumulating mutations that may eventually result in a more stable artemether-resistant haplotype that may subsequently spread, as reported for resistance to chloroquine (7, 35), pyrimethamine, or sulfadoxine (4). In vitro resistance is not synonymous with clinical failure, but it is on the critical path to eventual clinical failure.

Of further concern is the observation of an elevated  $IC_{50}$  for lumefantrine. Lumefantrine is an aryl alcohol, and there was evidence for cross-resistance of lumefantrine with halofantrine in French Guiana. We have included lumefantrine in the panel of antimalarials to be systematically assessed in current and future work. Again, the significance of this observation in terms of therapeutic efficacy is unclear, but it is a second warning for the long-term sustainability of the combination in this region. An assessment of the therapeutic efficacy of arte-

methem-lumefantrine is needed in the various endemicity areas across French Guiana.

This synopsis identified temporal trends pointing to changes in parasite in vitro susceptibility following changes in drug policy. Longitudinal assessment by the same laboratory with the same technology over a 12-year period provides a basis for deriving reasonably reliable temporal trends. The temporal dynamics of the IC<sub>50</sub>s have been somewhat variable from year to year, possibly reflecting some occasional geographic sample biases, but consistent trends are evidenced when a longer time frame is considered. In vitro resistance to multiple antimalarials is not specific to French Guiana. Multiple resistance has been reported in the neighboring countries of Suriname, Brazil, and Guyana. Our results highlight the usefulness of longitudinal in vitro susceptibility monitoring to document the consequences of a drug policy on resistance selection. In vitro assays provide invaluable information on emerging resistance against individual components of drug combinations such as quinine-doxycycline or artemether-lumefantrine. Surveillance capacities need to be strengthened urgently in the region in order to anticipate in good time the emergence of resistance and to prevent the spread of resistant lines across the region.

#### ACKNOWLEDGMENTS

We thank the colleagues who implemented and/or worked for the reference laboratory (T. Fandeur, J.B. Duchemin, J.M. Reynes, S. Laventure, and J.L. Sarthou). We are grateful to Cayenne's central hospital team, particularly B. Carne (Parasitology Unit) and F. Djossou (Health Center coordination), for organizing the collection of specimens.

This study was supported by the Prix Louis D. of the French Academy of Sciences and by the European Commission (contract QLK2-CT20021-1503-ResMalChip).

#### REFERENCES

- Brasseur, P., P. Bitsindou, R. S. Moyou, T. A. Eggelte, G. Samba, L. Penchenier, and P. Druilhe. 1993. Fast emergence of *Plasmodium falciparum* resistance to halofantrine. *Lancet* **341**:901–902.
- Brasseur, P., J. Kouamouo, R. S. Moyou, and P. Druilhe. 1990. Emergence of mefloquine-resistant malaria in Africa without drug pressure. *Lancet* **336**:59.
- Carne, B. 2005. Substantial increase of malaria in inland areas of eastern French Guiana. *Trop. Med. Int. Health* **10**:154–159.
- Cortese, J. F., A. Caraballo, C. E. Contreras, and C. V. Plowe. 2002. Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. *J. Infect. Dis.* **186**:999–1006.
- Dedet, J. P., P. Germanetto, G. Cordoliani, O. Bonnevie, and J. Le Bras. 1988. In vitro activity of various antimalarials (chloroquine, amodiaquine, quinine and mefloquine) against 32 isolates of *Plasmodium falciparum* in French Guiana. *Bull. Soc. Pathol. Exot. Filiales* **81**:88–93. (In French.)
- Demar, M., and B. Carne. 2004. *Plasmodium falciparum* in vivo resistance to quinine: description of two RIII responses in French Guiana. *Am. J. Trop. Med. Hyg.* **70**:125–127.
- Durrand, V., A. Berry, R. Sem, P. Glaziou, J. Beaudou, and T. Fandeur. 2004. Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) and their relationship to chloroquine resistance *in vitro*. *Mol. Biochem. Parasitol.* **136**:273–285.
- Esterre, P., G. Cordoliani, P. Germanetto, and Y. Robin. 1990. Epidemiology of malaria in French Guiana. *Bull. Soc. Pathol. Exot.* **83**:193–205. (In French.)
- Gruenfeld, J. 2001. *Plasmodium falciparum* chemoresistance in a village from French Guiana: comparison of in vivo tests on halofantrine-treated patients. M.D. thesis. Paris 7, Paris. (In French.)
- Hunt, P., A. Afonso, A. Creasey, R. Culleton, A. B. Sidhu, J. Logan, S. G. Valderramos, I. McNae, S. Cheesman, V. D. Rosario, R. Carter, D. A. Fidock, and P. Cravo. 2007. Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. *Mol. Microbiol.* **65**:27–40.
- Jambou, R., E. Legrand, M. Niang, N. Khim, P. Lim, B. Volney, M. T. Ekala, C. Bouchier, P. Esterre, T. Fandeur, and O. Mercereau-Puijalon. 2005. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. *Lancet* **366**:1960–1963.
- Kublin, J. G., J. F. Cortese, E. M. Njunju, R. A. Mukadam, J. J. Wirima, P. N. Kazembe, A. A. Djimde, B. Kouriba, T. E. Taylor, and C. V. Plowe. 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J. Infect. Dis.* **187**:1870–1875.
- Lakshmanan, V., P. G. Bray, D. Verdier-Pinard, D. J. Johnson, P. Horrocks, R. A. Muhle, G. E. Alakpa, R. H. Hughes, S. A. Ward, D. J. Krogstad, A. B. Sidhu, and D. A. Fidock. 2005. A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. *EMBO J.* **24**:2294–2305.
- Le Bras, J., and P. Deloron. 1983. In vitro study of drug sensitivity of *Plasmodium falciparum*: evaluation of a new semi-micro test. *Am. J. Trop. Med. Hyg.* **32**:447–451.
- Legrand, E., M. Demar, B. Volney, M. T. Ekala, M. Quinternet, C. Bouchier, T. Fandeur, C. Rogier, B. Carne, O. Mercereau-Puijalon, and P. Esterre. 2007. A first case of *Plasmodium falciparum* atovaquone-proguanil treatment failure in French Guiana. *Antimicrob. Agents Chemother.* **51**:2280–2281.
- Legrand, E., B. Volney, A. Lavergne, C. Tournegros, L. Florent, D. Accrombessi, M. Guillotte, O. Mercereau-Puijalon, and P. Esterre. 2005. Molecular analysis of two local falciparum malaria outbreaks on the French Guiana coast confirms the *msh1* B-K1/varD genotype association with severe malaria. *Malar. J.* **4**:26.
- Legrand, E., B. Volney, J. B. Meynard, P. Esterre, and O. Mercereau-Puijalon. 2007. Resistance to dihydroartemisinin *em*. *Infect. Dis.* **13**:808–809.
- Lepelletier, L., F. Gay, M. Nadire-Galliot, J. P. Poman, S. Bellony, J. Claustre, B. M. Traore, and J. Mouchet. 1989. Malaria in Guiana. I. General status of the endemic. *Bull. Soc. Pathol. Exot. Filiales* **82**:385–392. (In French.)
- Lial, J. P. 1999. Health assessments of a military company stationed on the Maroni River in French Guiana. *Med. Trop.* **59**:95–98. (In French.)
- Lim, P., S. Chy, F. Arie, S. Incardona, P. Chim, R. Sem, M. B. Denis, S. Hewitt, S. Hoyer, D. Socheat, O. Mercereau-Puijalon, and T. Fandeur. 2003. *pfrt* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. *Antimicrob. Agents Chemother.* **47**:87–94.
- Liu, D. Q., R. J. Liu, D. X. Ren, D. Q. Gao, C. Y. Zhang, C. P. Qui, X. Z. Cai, C. F. Ling, A. H. Song, and X. Tang. 1995. Changes in the resistance of *Plasmodium falciparum* to chloroquine in Hainan, China. *Bull. W. H. O.* **73**:483–486.
- Maisonneuve, H., F. Joly, M. John, G. Carles, and J. F. Rossignol. 1988. Efficacy of halofantrine in *Plasmodium falciparum* or *Plasmodium vivax* malaria in a resistance area (French Guiana). *Presse Med.* **17**:99–102. (In French.)
- Mita, T., A. Kaneko, J. K. Lum, B. Bwijo, M. Takechi, I. L. Zungu, T. Tsukahara, K. Tanabe, T. Kobayakawa, and A. Bjorkman. 2003. Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *Am. J. Trop. Med. Hyg.* **68**:413–415.
- Newton, P. N., R. McGready, F. Fernandez, M. D. Green, M. Sunjio, C. Bruneton, S. Phanouvong, P. Millet, C. J. Whitty, A. O. Talisuna, S. Proux, E. M. Christophel, G. Malenga, P. Singhasivanon, K. Bojang, H. Kaur, K. Palmer, N. P. Day, B. M. Greenwood, F. Nosten, and N. J. White. 2006. Manslaughter by fake artesunate in Asia—will Africa be next? *PLoS Med.* **3**:e197.
- Noronate, N., R. Durand, A. Tall, L. Marrama, A. Spiegel, C. Sokhna, B. Pradines, S. Cojean, M. Guillotte, E. Bischoff, M.-T. Ekala, C. Bouchier, T. Fandeur, F. Arie, J. Patarapotikul, J. Le Bras, J. F. Trape, C. Rogier, and O. Mercereau-Puijalon. 2007. Rapid dissemination of *Plasmodium falciparum* drug resistance despite strictly controlled antimalarial use. *PLoS ONE* **1**:e139.
- Pradines, B., C. Rogier, T. Fusai, A. Tall, J. F. Trape, and J. C. Doury. 1998. In vitro activity of artemether against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs. *Am. J. Trop. Med. Hyg.* **58**:354–357.
- Reed, M. B., K. J. Saliba, S. R. Caruana, K. Kirk, and A. F. Cowman. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**:906–909.
- Reynes, J. M., J. Fargette, P. Gaborit, and S. Yarde. 1997. In vitro responses of *Plasmodium falciparum* isolates to five antimalarial drugs in French Guiana during 1994 and 1995. *Mem. Inst. Oswaldo Cruz* **92**:251–252.
- Sarthou, J. L., and J. M. Reynes. 1996. Malaria in French Guiana: chemoresistance of *Plasmodium falciparum* isolates. *Rev. Epidemiol. Sante Publique* **44**:42. (In French.)
- Schaapveld, K. 1984. Integration on antimalaria activities into basic health services in Suriname. ICG Printing, Dordrecht, The Netherlands.
- Sidhu, A. B., S. G. Valderramos, and D. A. Fidock. 2005. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Mol. Microbiol.* **57**:913–926.
- Sidhu, A. B., D. Verdier-Pinard, and D. A. Fidock. 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfrt* mutations. *Science* **298**:210–213.
- Walliker, D., P. Hunt, and H. Babiker. 2005. Fitness of drug-resistant malaria parasites. *Acta Trop.* **94**:251–259.

34. Wang, X., J. Mu, G. Li, P. Chen, X. Guo, L. Fu, L. Chen, X. Su, and T. E. Wellems. 2005. Decreased prevalence of the *Plasmodium falciparum* chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against *P. falciparum* malaria in Hainan, People's Republic of China. *Am. J. Trop. Med. Hyg.* **72**:410–414.
35. Wootton, J. C., X. Feng, M. T. Ferdig, R. A. Cooper, J. Mu, D. I. Baruch, A. J. Magill, and X. Z. Su. 2002. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature* **418**:320–323.
36. Zalis, M. G., L. Pang, M. S. Silveira, W. K. Milhous, and D. F. Wirth. 1998. Characterization of *Plasmodium falciparum* isolated from the Amazon region of Brazil: evidence for quinine resistance. *Am. J. Trop. Med. Hyg.* **58**:630–637.